

## Cephalosporin C Acylase from Microbes for One-step Enzymatic Transformation of Cephalosporin C to 7-Aminocephalosporanic Acid

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Cephalosporins are the  $\beta$ -lactam antibiotics for the treatment of infection diseases caused by gram-positive and gram-negative bacteria. Fungus *Cephalosporium acremonium* produces cephalosporin C (CPC) that is not potent for clinical use. Its molecule can be transformed to 7-amino cephalosporanic acid (7-ACA) as the intermediate compound for making semi-synthetic cephalosporin derivates. The first method for production of 7-aminocephalosporanic acid involves the chemical reaction using toxic reagents and laborious procedures. The second method uses two-step and one-step enzymatic transformation. In two-step method, the first step involves the conversion of Ceph-C to Glutaryl-7-amino cephalosporanic acid (GL-7-ACA) by D-amino acid oxidase (DAO) and the second step, GL-7-ACA acylase (GA) hydrolyzes GL-7- ACA to produce 7-ACA. The two-step enzymatic transformation was used widely because of its safety and environmental friendliness. The one-step enzymatic transformation is developed because of process simplification and cost reduction. This method uses cephalosporin C acylase for transformation CPC to 7-ACA. Same microbes can produce cephalosporin C acylase such as *Achromobacter xylosoxidans*, *Aeromonas* sp., *Arthrobacter viscosus*, *Bacillus laterosporus*, *Flavobacterium* sp., *Paecilomyces* sp., and *Pseudomonas* sp. The natural of cephalosporin C acylase catalyzed the CPC to 7-ACA directly in a very low efficiency and the protein engineering of cephalosporin C acylase was used to increase activity.

**Keywords:** Cephalosporin C, 7-aminocephalosporanic acid, D-amino acid oxidase, GL-7-ACA acylase, cephalosporin C acylase, *Cephalosporium acremonium*.

Cephalosporin C (CPC) was the second  $\beta$ -lactam antibiotics to be discovered after penicillin from the fungus *Cephalosporium acremonium* by Giuseppe Brotzu<sup>1,2</sup>. CPC showed a moderate antibacterial activity: minimum inhibitory concentration values were in the range of 25–100 and 12– 25  $\mu$ g/mL for gram-positive and gram-negative bacteria, respectively<sup>2</sup> and is not potent for clinical use<sup>3</sup>. Robert Morin discovered a process by which the D- $\alpha$ - amino adipoyl side chain of

CPC could be removed, generating 7-aminocephalosporanic acid (7-ACA) in 1962<sup>2</sup>. 7-ACA is the intermediate for semi-synthetic cephalosporin derivates. Some semi-synthetic cephalosporin such as cefezole, cefotiam, cefazedone, and cefazolin were produced by 7-ACA<sup>4</sup>.

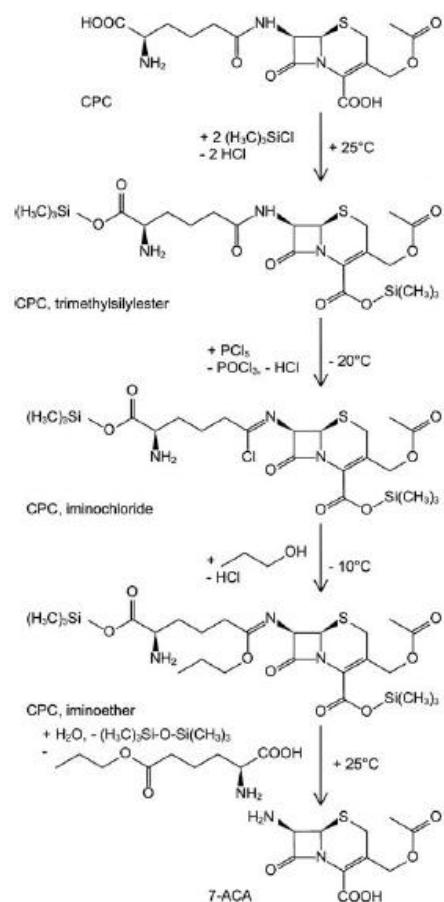
The cephalosporin antibiotics are divided into two groups; the first is derived from penicillin (G or V) and the second from CPC. The most products that have intrinsic oral activity are better made from penicillin, while the most products made from CPC are insufficiently absorbed from the human gut to be therapeutically adequate by the

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oral route, unless converted to pro-drugs by esterification<sup>5</sup>. The cephalosporins are clinically active against gram-positive as well as gram-negative organisms. Cephalosporins are broad-spectrum, less toxic and resistant to degradation of  $\beta$ -lactamase as compared to penicillins<sup>1,3</sup>.

#### Conversion CPC to 7-ACA

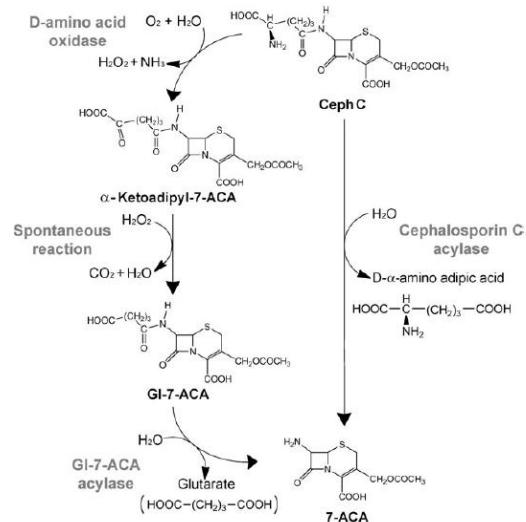
Cephalosporins derivates are semi-synthetic products that derive from the fermentative product CPC. CPC is initially converted to 7-ACA by either a chemical or an enzymatic catalysis removal of the 7-amino adipoyl side chain. The chemical catalysis was developed by Morin *et al.* and this process is used to 7-ACA production in an industrial scale (Scheme 1)<sup>5</sup>. Enzymatic transformation of CPC to 7-ACA is the best alternative and has industrial significance<sup>3</sup>.



**Scheme 1.** Reaction scheme of chemical cleavage of CPC into 7-ACA<sup>5</sup>

The chemical method has been replaced by enzymatic method for producing 7-ACA because of (1) safety and environmental contamination: dangerous and toxic reagents (i.e., trimethylchlorosilane, phosphorus pentachloride, dichloromethane, dimethylaniline, etc.) are absent in the enzymatic transformation<sup>1,2</sup>; (2) selectivity: owing to enzyme selectivity, the use of permanent or temporarily protective groups is avoided<sup>2</sup>; (3) energy consumption: the chemical process uses low temperatures and exothermic steps, while the enzymatic approach is carried out at a fixed temperature of 20–30°C<sup>2</sup>; (4) equipment: owing to the use of aggressive reagents, the chemical process requires glass-lined vessels and dedicated and expensive equipment to transport and charge these materials, to trap spilling or vapors, etc.<sup>2</sup>; and (5) quality: in the enzymatic 7-ACA preparations, the levels of oligomers, 7-desacetoxycephalosporanic acid, and desacetyl-7-ACA (or corresponding lacton) are considerably lower—the typical assay is 3–6 % higher than for the chemical process<sup>2</sup>.

The biocatalytic conversion for CPC into 7-ACA, the key intermediate to cephalosporin derivates was developed<sup>5</sup>. CPC can be transformed by two-step enzymatic method by D-amino acid oxidase (DAAO) and glutaryl acylase (GAC) or One-step enzymatic method by a CPC acylase (CA)<sup>1,2,5,7,8</sup>.



**Scheme 2.** Bioconversion of cephalosporin C to 7-ACA<sup>2</sup>

The most widely used enzymatic approach is represented by a two-step enzymatic method that employs DAAO (D-amino acid oxidase) and Gl-7-ACA acylase (Scheme 2)<sup>5</sup>. The flavoenzyme D-amino acid oxidase (DAAO; EC 1.4.3.3) catalyzes the oxidative deamination of neutral and basic D-amino acids but not acid D-amino acid, which are substrates of D-aspartate oxidase<sup>9</sup>. D-Amino acid oxidases are rather frequent in nature; they are present in mammalian organs, mainly in the kidneys. Different microbes too are known to produce DAAO, such as the yeasts *Candida tropicalis*<sup>5</sup>, *Rhodotorula gracilis*<sup>5</sup>, and *Trigonopsis variabilis*<sup>5</sup>, the fungi *Fusarium oxysporum*<sup>5</sup>, *Fusarium solani*<sup>5</sup>, *Neurospora crassa*<sup>5</sup>, *Penicillium chrysogenum*<sup>5</sup> and *Rhodosporidium* sp.<sup>5</sup>; prokaryotic microbial *Arthrobacter protophormiae*<sup>5</sup>, *Rubrobacter xylanophilus*<sup>9</sup>, and *Streptomyces coelicolor*<sup>5</sup>. Only two enzymes, namely DAAO from *Rhodotorula gracilis* and from *Trigonopsis variabilis*, have been developed into an industrial biocatalyst<sup>5</sup>.

GAs (glutaryl acylases; EC 3.5.1.93) have been classified into five classes on the basis of their sequence, molecular mass and enzymatic properties. Members of each class are very similar on the basis of substrate specificity and sequence conservation (they share >90% of nucleic acid or amino acid sequence identity). Members of class I (e.g. P130 from *Pseudomonas* sp. 130) and class III (e.g. N176 from *Pseudomonas* sp. N176) show the highest activity to CPC (4% relative to Gl-7ACA)<sup>10</sup>.

Glutaryl acylase (GA) is a metal free heterodimer of 16 kDa+54 kDa subunits without any prosthetic group. This enzyme has been isolated from many organisms, but only the one from *Pseudomonas diminuta* was cloned and expressed in a recombinant *E. coli* and has been developed into an industrial biocatalyst<sup>5</sup>. GA activity has been reported in bacteria from *Aeromonas*<sup>2</sup>, *Arthrobacter*<sup>2</sup>, *Bacillus*<sup>2</sup>, and *Flavobacterium*<sup>2</sup>, *Pseudomonas*<sup>2,5</sup>, and from the fungus *Paecilomyces*<sup>2,3</sup>.

The alternative enzymatic approach is a one-step enzymatic method process in which CPC is converted directly into 7-ACA by a CPC acylase (CA; Scheme 2). As shown in Scheme 2, acylases can cleave the amide bond through which the cepham nucleus is linked to the acyl side chain<sup>2</sup>. Cephalosporin acylase (CA, EC 3.5.1.11) is a

member of the N-terminal nucleophile (Ntn) hydrolase superfamily, in which the precursor gene is translated into a single polypeptide chain and then folded into a self-activating pre-protein activated by intra-molecular double cleavages. The first cleavage is most important for generating the active center catalysis residue (Ser) of the enzyme at the N terminus of the  $\beta$ -subunit. The second cleavage is also essential for activating the enzyme, forming a mature heterodimer with the  $\alpha$ - and  $\beta$ -subunits and releasing a spacer peptide of different lengths (8–11 aa) in varying CAs. According to the diversity in substrate specificity, CAs can be divided into two classes, the cephalosporin C (CPC) acylase and the glutaryl-7-aminocephalosporanic acid (GL-7-ACA) acylase classes. Both enzymes classes form 7-aminocephalosporanic acid (7-ACA)<sup>11</sup>.

Sonawane (2006) classified CA base on their substrate (CPC, GL-7-ACA, and GL-7-ADCA) specificity. The types of cephalosporin acylase are: type 1 is true cephalosporin C acylase. CPC acylase can hydrolyze only CPC and is not active for GL-7-ACA; type 2 GL-7-ACA acylase. This enzymes can hydrolyze GL-7-ACA to 7-ACA in high yield but have less activity against CPC; type 3 GL-7-ADCA acylase. Their enzymes have activity against GL-7-ADCA but their activity against GL-7-ACA is not known<sup>12</sup>.

Recently, the CPC acylase is very attractive at industrial level because it possesses very simple with reducing cost, although its catalytic performance, including its activity and product inhibition resistance, is not yet as satisfactory as that of the GL-7-ACA acylase<sup>2,11</sup>. Some studies focused on identifying more active enzymes from different microbial sources, such as *Achromobacter xylosoxidans*<sup>12</sup>, *Aeromonas* sp.<sup>1,12,13</sup>, *Alcaligenes xylosoxidans* MTCC\*491<sup>1,13</sup>, *Arthrobacter viscosus*<sup>1,12</sup>, *Bacillus megaterium*<sup>1</sup>, *Bacillus laterosporus*<sup>12</sup>, *Bacillus* sp.<sup>1</sup>, *Flavobacterium* sp.<sup>12,13</sup>, *Micrococcus luteus* NCIM 2170<sup>8</sup>, *Paecilomyces* sp.<sup>12</sup>, *Pseudomonas diminuta* N176<sup>1,2,7,11</sup>, *Pseudomonas cepacia*<sup>12</sup>, *Pseudomonas nitroreducens*<sup>12</sup>, *Pseudomonas paucimobilis*<sup>12</sup>, *Pseudomonas putida*<sup>12</sup>, *Pseudomonas* sp. 130<sup>11</sup>, *Pseudomonas* sp. SE83<sup>1,7</sup>, *Pseudomonas* sp. V22<sup>2</sup>.

The natural of cephalosporin C acylase catalyzed the CPC to 7-ACA directly in a very low efficiency<sup>14,15,16</sup>, significant substrate inhibition<sup>14</sup>,

and product inhibition<sup>14</sup>. In recent years, protein engineering of cephalosporin C acylase was developed to overcome the above limitations<sup>14, 15</sup>. Protein engineering approach based on combined use of error-prone PCR mutagenesis, molecular modeling, site saturation and site-directed mutagenesis<sup>17</sup> were used to enhance the catalytic efficiency of CPC acylase. Wang et al. found a mutant of CPC acylase activity from *Pseudomonas diminuta* N176, Ala675Gly showed 11.2-fold compared with its wild type<sup>7</sup>; Golden et al. found two mutants of cephalosporin acylase from *Pseudomonas* N176, H57 $\alpha$ S/H70 $\beta$ S and M165 $\alpha$ S/H57 $\beta$ S/H70 $\beta$ S had  $V_{max}$  4.2- and 6.0-fold compared with wild type, respectively<sup>10</sup>; Zhang et al. found two mutants of CPC acylase from *Pseudomonas* sp. SE83, mutant protein D2 (227-AM-228 deletion) and D4 (212-ADLA-215 deletion) had  $K_{cat}/K_m$  values 1.46- and 2.02-fold compared with the original control, respectively<sup>11</sup>; Xiao et al. found two mutants of CPC acylase form *Pseudomonas* SE83, mutant H57 $\beta$ A/H70 $\beta$ Y and mutant H57 $\beta$ A/H70 $\beta$ Y/I176 $\beta$ N had  $K_{cat}/K_m$  values 1.2- and 5.6-fold compared with the original control, respectively<sup>14</sup>; Pollegiani et al. found two mutants of the CPC acylase from *Pseudomonas* N176, M31 $\beta$ F/H57 $\beta$ S/H70 $\alpha$ S and A215 $\alpha$ Y/M31 $\beta$ F/H70 $\beta$ S showed 3.3- and 4.3 fold improvements in  $V_{max}$  on CPC compared with its starting template (M31 $\beta$ F), respectively with eliminated substrate inhibition and reduced product inhibition<sup>14</sup>; A mutant of the CPC acylase acyII from *Pseudomonas* SE83, V122 $\alpha$ A/G140 $\alpha$ S/F58 $\beta$ N/I75 $\beta$ T/I176 $\beta$ V/S471 $\beta$ C (S12) showed a 7.5- and 3.7-fold improvement in the specific activity and  $K_{ip}$ , respectively, compared with the wild type<sup>14</sup>; Ishii et al. mutated Met269 and Ala271 of cephalosporin C acylase can increase activity 1.6-fold and 1.7-fold respectively<sup>15</sup>; Ren et al. found a mutant of the cephalosporin acylase from gene *acyII* in *Pseudomonas* sp., G139 $\alpha$ S/F58 $\beta$ N/I176 $\beta$ V/S471 $\beta$ C showed 2.86-fold improvement in  $K_{cat}/K_m$  compared with wild type<sup>14</sup>; Oh et al. found that the deacylation activity of the mutation Q50 $\beta$ M-Y149 $\alpha$ K-F177 $\alpha$ G toward CPC improved by 7.9-fold<sup>16</sup>.

## CONCLUSION

- The biotransformation of cephalosporin C to 7-ACA in one step enzymatic with cephalosporin

acylase is a simple process with low cost and potential to applied in industrial production.

- The protein engineering of cephalosporin C acylase was developed to increase its activity and stability of CPC acylase.

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